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NEW MILBEMYCIN DERIVS. + USEFUL AS INSECTICIDES, ACARICIDES, EMATICIDES AND ANTHELMINTICS

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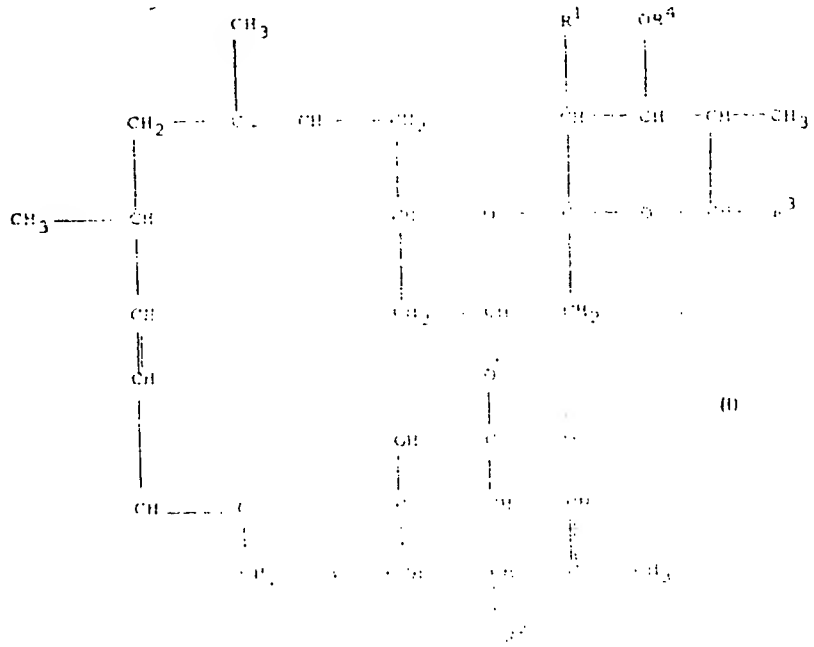
(54) **Pesticidal and anthelmintic milbemycins**

(57) Compounds of formula (I):



(I)

wherein R^1 is hydroxy, R^2 is hydrogen or methyl, R^3 is methyl, and R^4 is 2,4-dimethylpentanoyl or 2,4-dimethylpent-2-enoyl, have pesticidal and anthelmintic activity

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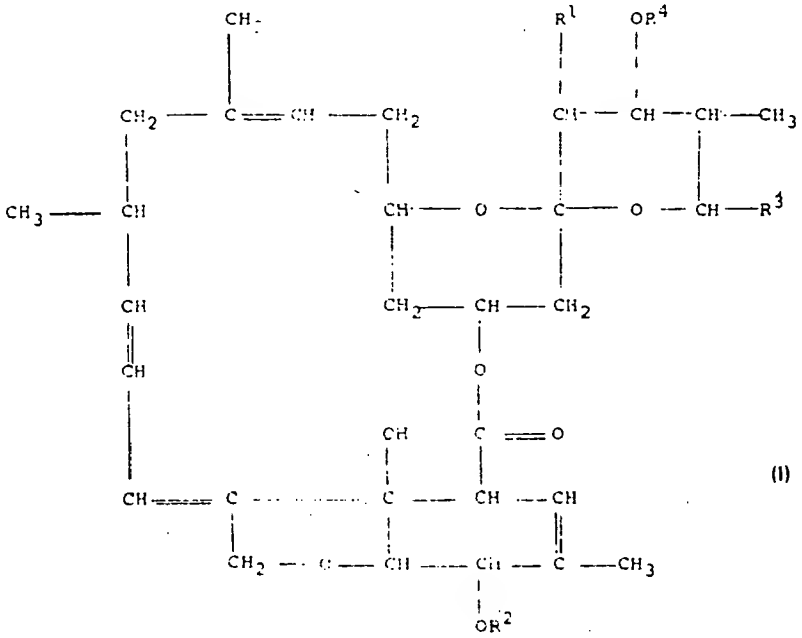
SPECIFICATION

Macrocyclic lactones

This invention relates to novel compounds and to their use as pesticides and anthelmintics. The milbemycins are a group of macrocyclic lactones obtained from the fermentation of microorganisms for example as described in US Patent 3,984,564.

We have now discovered that fermentation of certain organisms under specified conditions gives rise to a number of milbemycin species not previously described.

The present invention provides a compound of formula (I) :



(I)

wherein

(a) R^1 is hydroxy, R^2 is hydrogen or methyl, R^3 is methyl, and R^4 is 2,4-dimethylpentanoyl or 2,4-dimethylpent-2-enoyl; or

(b) R^1 is hydroxy, R^2 is hydrogen or methyl, R^3 is methyl, and R^4 is 2-methylbutanoyl; or

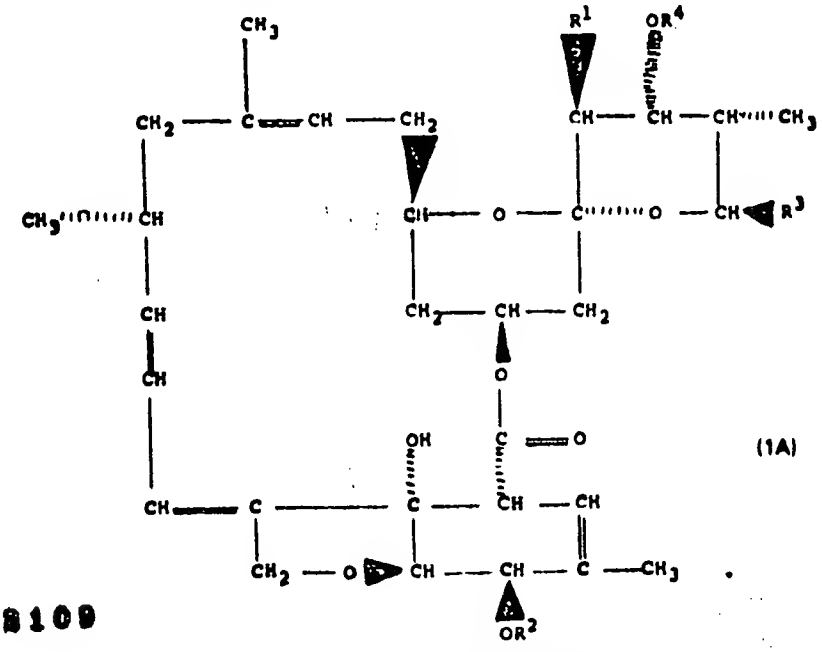
(c) R^1 is hydroxy, R^2 is hydrogen, R^3 is ethyl, and R^4 is 2,4-dimethylpentanoyl; or

(d) R^1 and R^4 are both hydrogen, and R^2 and R^3 are both methyl.

Particular compounds according to the invention are those set out in Table 1 below wherein the meanings of R^1, R^2, R^3 and R^4 in Formula I are given for each compound.

Compound No.	R^1	R^2	R^3	R^4
1	OH	CH_3	CH_3	$-\overset{O}{\overset{\|\|}{C}}-\overset{CH_3}{\overset{\|}{CH}}-CH_2-CH_3$
2	OH	CH_3	CH_3	$-\overset{O}{\overset{\|\|}{C}}-\overset{CH_3}{\overset{\|}{C}}=CH-\overset{CH_3}{\overset{\|}{CH}}-CH_3$
3	H	CH_3	CH_3	H
4	OH	H	CH_3	$-\overset{O}{\overset{\|\|}{C}}-\overset{CH_3}{\overset{\|}{C}}=CH-\overset{CH_3}{\overset{\|}{CH}}-CH_3$
5	OH	H	CH_3	$-\overset{O}{\overset{\|\|}{C}}-\overset{CH_3}{\overset{\|}{CH}}-CH_2-\overset{CH_3}{\overset{\|}{CH}}-CH_3$
6	OH	CH_3	CH_3	$-\overset{O}{\overset{\|\|}{C}}-\overset{CH_3}{\overset{\|}{CH}}-CH_2-\overset{CH_3}{\overset{\|}{CH}}-CH_3$
7	OH	H	CH_2CH_3	$-\overset{O}{\overset{\|\|}{C}}-\overset{CH_3}{\overset{\|}{CH}}-CH_2-\overset{CH_3}{\overset{\|}{CH}}-CH_3$
8	OH	H	CH_3	$-\overset{O}{\overset{\|\|}{C}}-\overset{CH_3}{\overset{\|}{CH}}-CH_2-CH_3$

The compounds of formula I are believed to be isolated as single isomers having a particular stereochemistry as represented by formula (IA) :



(1A)

This was determined by analogy with the absolute stereochemistry for known milbemycins given for example in J. Antibiotics *33* (10), 1121. However, the invention herein relates to the compounds actually obtained by fermenting the microorganism and as defined hereinafter by various physical and chemical parameters and is not intended to be limited to the particular stereochemical isomers shown in formula (IA).

The compounds of formula (I) are prepared by fermentation techniques. Further according to the present invention there is provided a process for preparing a compound of formula (I) which process comprises cultivating a strain of Streptomyces and isolating the compound of formula (I) from the fermentation mixture.

A suitable strain of streptomyces is the b41-146 strain (fully described in US Patent 3,984,564) of a *Streptomyces* species which has been deposited at the Northern Research Laboratory, US Department of Agriculture, Peoria, Illinois, USA under deposit number NRRL 5739, and at the National Collection of Industrial and Marine Bacteria, 135 Abbey Road, Aberdeen, Scotland under deposit number NCIB 11876, or a mutant thereof.

The fermentation procedure and isolation techniques are set out below in Example 1.

The compounds are useful as pesticides, particularly as insecticides, acaricides and nematicides, and as anthelmintics.

They are suitably administered to the pest or its environment in the form of a composition comprising the compound of formula (I) and an appropriate carrier or diluent. Such compositions form a further aspect of the invention.

The compositions are suitably formulated in a conventional manner depending upon the intended use. Thus for use as insecticides, acaricides or nematocides, they can be applied in any of the forms conveniently employed in agriculture. Thus in a further aspect, the invention provides an insecticidal composition comprising a compound of formula (I) as hereinbefore defined or a salt thereof in combination with an agriculturally acceptable carrier.

Depending on the intended use, one or more compounds of formula (I) are either dissolved or dispersed in a suitable liquid vehicle or admixed with or adsorbed on a suitable solid vehicle and the resulting composition is made available in any of such forms as emulsifiable concentrate, oil, wetter powder, dusts, granules, tablets, aerosol mist, ointment, etc. There may also be added to these preparations such additives as emulsifers, suspending agents, extenders, penetrants, wetting agents, thickners or stabilizers, as necessary. These preparations can be pruduced by the known methods.

The proportion of the compound of formula (I) in such an insecticidal/acaricidal preparation depends on the intended use and application.

For use as an enthelmintic, the compound of formula (I) is suitably combined with a pharmaceutically acceptable carrier. The composition thus formed can be suiteble for oral or parenteral administration as is known in the art. The dosage employed will depend upon the animal being treated and the severity of the conditions.

The following examples illustrate the invention.

Example 1

The organism used was a *Streptomyces* strain identified as NCIB 11876 which has been deposited with the National Collection of Industrial and Marine Bacteria, 135 Abbey Road, Aberdeen AB9 8DG, Scotland.

An innoculum was grown on an Oatmeal Agar slope, streaked 20 days earlier at 28°C. 10 ml of a Starter seed medium (Y. Takiguchi *et al* Journal of Antibiotics, October 1980, p1120) was used to dislodge spores and mycelium from those slopes and the resulting suspension was pipetted out into 2 fresh 250 ml Erlenmeyer flasks containing 25 ml of starter seed medium. These were incubated on an orbital shaker at 200 rpm at 28°C for 2 days before 20 aliquots of 5 ml were used to innoculate 20 250 ml flasks each containing 25 ml of seed medium. After 2 days shaking as before, these were used to innoculate a 14 l capacity "Microferm" (New Brunswick Scientific (UK) Ltd) fermentation vessel containing 9 l of fermentation medium (Y. Takiguchi *et al* Journal of Antibiotics, October 1980, p1120). Aeration was at 8 l of air/minute supplemented by stirring at 400 rpm at 28°C with polypropylene glycol (25 ml) acting as an inert antifoaming agent. The grow was harvested after 7 days by centrifugation at 3000 rpm for 10 minutes (Damon/IEC PR-6000) to separate the plentiful actinomycete pellets. At harvest, the whole broth was a deep yellow colour.

The centrifuged mycelium was taken and allowed to stand ovarnight at room temperature with 2 l of methanol. After filtration, the solid residue was re-extracted twice with methanol (1 litre) and all the aqueous methanol extracts were combined. The resulting solution (approx 5 l) was concentrated *in vacuo* to approximately 1.5 l before partitioning with hexane (2 portions of 1.5 l). The hexane phases were concentrated to dryness and redissolved in methanol (400 ml) which was stored overnight at −20°C. The resulting precipitate was removed by filtration, the filtrate was concentrated to yield a brown oil (12.22g).

The brown oil (10g) was applied to a column of Kieselgel 60 (E. Merck, Darmstadt) (25 cm by 3 cm) which was successively eluted with hexane : acetone 95:5 (1 litre), 90:10 (1.3 litre) and 80:20 (1 litre) followed by acetone (0.5 litre) and finally methanol (0.5 litre) to give 18 fractions.

Fraction 12 (400 mg) was chromatographed on silica-gel 60-F254 preparative plates (0.25 mm) developed in chloroform:ethyl acetate 3:1 to give bands discernible by their quenching of gel fluorescence under short wave UV radiation. These areas of silica were removed from the plates and eluted with methanol to yield five fractions; 12A (38 mg, Rf=0.53), 12B (16 mg, Rf=0.47), 12C (31 mg, Rf=0.38), 12D (73 mg, Rf=0.29) and 12E (72 mg, Rf-0.26). HPLC showed fractions 12A to 12C contained compounds of interest and so portions of each were further purified by semi-preparative reverse phase HPLC (using a HICHROM S5ODS-2 column [150 mm by 8 mm[eluted with 2.5 ml·min of 85% methanol water using UV detection at 246 nm) to provide pure samples of milbemycins designated as compounds 1, 2, 3 and 6. These were each analysed by electron impact and chemical ionisation (the reagent gas was ammonia) mass spectrometry on a Finnigan-Mat 8200 mass spectrometer (Table B) and UV (in methanol using a Pye Unicam instrument) (Tabie A); these techniques revealed the compounds to be unlike any previously reported.

The accurate masses of the molecular ions of compounds 1-3 and 6 were determined by high resolution mass spectrometry (Finnigan-Mat 8200 peak matching in comparison with perfluorokerosene [PFK] the results are shown in Table C along with the corresponding empirical formulae. The compounds were also investigated by 400 MHz NMR (Jeol GX400), the chemical shift data are shown in Tables D and E. From the combination of this information it was possible to assign the structures for compounds 1 and 2. There was insufficient material for confirmation of the structure of compound 3 but the mass spectral evidence indicates it to have the structure shown in Table I.

Compound 6 was identical by mass spectrometry to the previously reported alpha b (Takiguchi, Mishima, Okuda and Terao; Journal of Antibiotics, 1980, 33 (10), 1120 1127). However, careful interpretation of the 400 MHz NMR proton spectrum showed the presence of 6 methyl doublets (at delta 1.01 [c12-Me], 0.83 [C24-Me], 1.22 [C26-Me], 1.17 [C21-Me], 0.88 and 0.91 [C-5-Methyls] where only four doublets and one triplet would be expected for alpha 6, COSY 2D-NMR allowed the full assignment of the spectrum shown in Tables D and E; corresponding to the structure shown in Table I.

Fraction 14 (1017 mg) from the original silica column was further purified on a LOBAR normal phase silica column (E MERCK) eluted with hexane:ethanol 96:4 and 94:6 using UV detection at 246 nm. to yield 80 fractions. These were analysed by TLC (Silica gel 60-F254, eluted with hexane-acetone 65:35, compounds were visualised by their quenching of gel fluorescence under UV light at 254 nm and by the blackish coloured spots on a yellow background generated with "phosphomolybdate spray reagant". The fractions were also examined by anaytical HPLC (SpectraPhysics SP8100 with HICHROM S50DS-2 [250 by 4.9 mm] at 40°C eluted with 1 ml/min of a programmed solvent gradient ranging from methanol:water 83:17 to 100% methanol over 35 minutes [15 mins isocratic followed by a linear gradient). Under these conditions, the compounds have the following retention times : 1 - 10 min, 2 - 12.6 min, 3 - 7.8 min, 4 - 10.0 min, 5 - 11.9 min, 6 - 15.6 min and 7 - 15.2 min). Appropriate fractions (181 mg) were combined prior to preparative TLC on 4 silica gel 60-F254 preparative plates (2.5 mm). The developing solvent was chloroform:acetone 3:1. Fluoroescence quenching bands at Rf - 0.23, 0.46, 0.65 and 0.96 were removed and eluted with ethyl acetate (2 by 50 mls) to give four fractions One of these was shown to contain significant quantities of compounds of interest which were cleaned up by semi-preparative HPLC to yield three pure compounds designated 4, 5 and 7. Analysis as before gave the mass spectral, UV and NMR data shown in tables B, A, D and E and allowed the assignment of structures given in Table I. Again, with compounds 5 and 7, mass spectral evidence suggested that the materials were the previously reported alphas 5 and 7 but the NMR confirmed the presence of the terminal iso-propyl group on the C-23 ester chain. Decoupling experiments on the protons coupling to some of the methyl groups of compound 5 confirmed the assignments. No trace of the straight chain compounds could be found.

A repeat grow of this microorganism resulted in the isolation of larger quantities of novel compounds 1, 2, 4 and 5. In addition, a further novel compound designated 8 has been isolated and identified by nmr, UV and mass spectral data.

TABLE A

Compound No.	*UV absorbance maxima (nm) methanolic solution*		
1	237 (sh)	244	251 (sh)
2	236	244	252 (sh)
3	237	244	252 (sh)
4	238 (sh)	246	254 (sh)
5	239 (sh)	246	253 (sh)
6	238 (sh)	246	253 (sh)
7	238 (sh)	246	253 (sh)
8	237 (sh)	244	253 (sh)

TABLE B

Compound No.	Mass Spectral Data - fragment ions (M^+)						
1	658	640	458	414	396	264	246
	195	167	151	125			
2	684	666	458	414	396	264	246
	195	167	151	125	111		
3	558	540	522	398	380	248	229
	197	179	169	161	151	125	
4	670	652	560	524	444	427	414
	396	278	264	195	167	151	111
5	672	264	562	524	444	427	414
	396	278	264	195	167	151	85
6	686	668	458	414	396	264	246
	195	167	151	125	85		
7	686	668	576	444	428	410	293
	278	248	209	181	151	85	
8	644	626	608	534	444	427	414
	396	195	167	151			

TABLE C

Compound No.	Mass Spectral Data : Accurate Mass (M^+)	Calculated Molecular Formula
1	658.371812	$C_{37}H_{54}O_{10}$
2	684.387596	$C_{39}H_{56}O_{10}$
3	558.319057	$C_{33}H_{46}O_8$
6	686.4042	$C_{39}H_{58}O_{10}$

Nuclear Magnetic Resonance data are given in the following two tables. The location of the relevant protons is given according to the numbering of the carbon atoms in them Ilbemycin skeleton as follows :

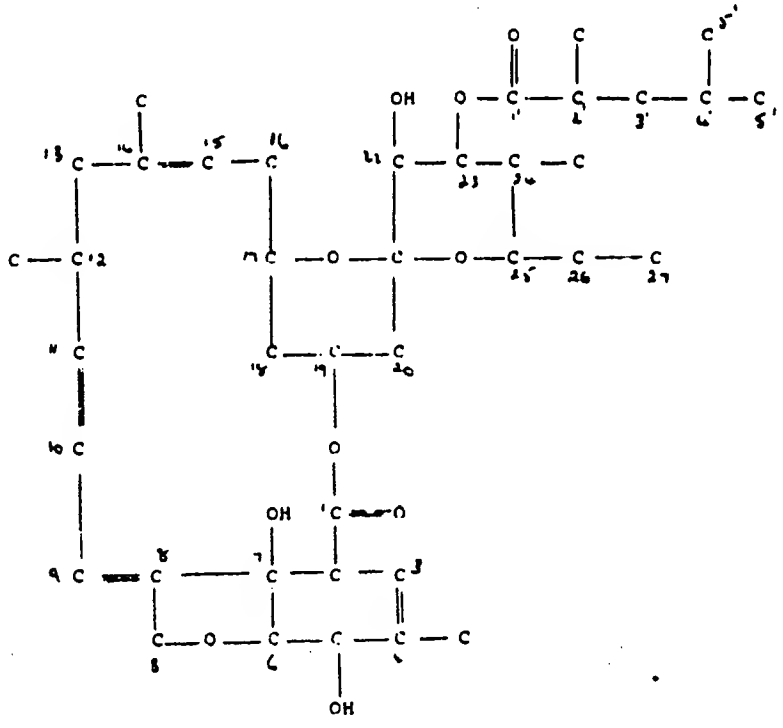


TABLE D

Location of Protons (C-number)	Nmr Data ($CDCl_3$, Standard - tetramethylsilane) δ, for Compound No.						
	1	2	4	5	6	7	8
2	3.32	3.32	3.28	3.28	3.32	3.28	3.26
3	5.39	5.39	5.41	5.40	5.38	5.40	5.39
4 (CH_3)	1.82				1.82	1.88	1.87
5(H.CH_3)	3.97.3.52	3.98.3.52	4.29.-	4.29,2.32	3.97.3.52	4.29,2.34	4.28,2.32
5	4.03	4.03	3.96	3.96	4.04	3.96	3.95
7 (OH)	4.00	4.02	4.68	3.94	4.00	3.94	3.93
3	4.66	4.65	5.78	4.68	4.66	4.68	4.66
9	5.77	5.77	5.74	5.79	5.76	5.80	5.76
10	5.74	5.74	5.74	5.75	5.74	5.75	5.74
11	5.35	5.34	5.37	5.35	5.38	5.36	5.55
12 (H.CH_3)	2.43.1.00	2.43.-	2.43.1.01	2.42.1.00*n	2.43-1.01	2.42,0.99	
13 (a,e)				1.86,2.21		1.85,2.21	
14 (CH_3)				1.54	1.54	1.53	1.52
15	4.97	4.97	4.97	4.96	4.96	4.94	4.96
16	2.23			2.22	2.22	2.22	2.22

TABLE E

Nmr Data (CDCl$_3$, Standard - tetramethylsilane) δ, for Compound No.

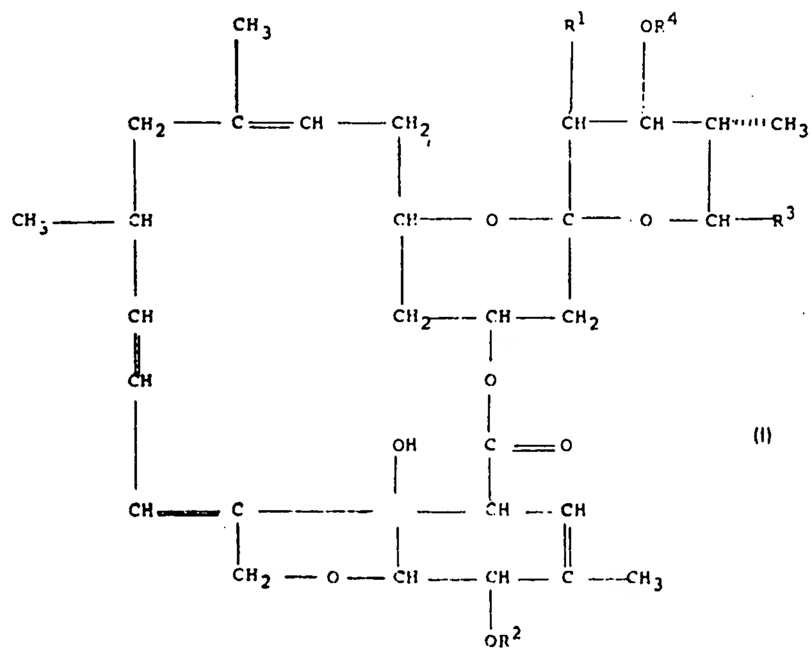
Location of Protons (C-number)	for Compound No. 1	2	4	5	6	7	8
17	3.58			3.60	3.60	3.60	3.59
18 (a,e)				0.92,1.82	0.92,1.82	0.90,1.82	
19	5.32	5.33	5.34	5.32	5.32	5.31	5.31
20 (a,e)				-,1.91		0.1.90	
22 (H,OH)	3.20,-			3.20,1.88	3.22	3.20,1.88	3.20-
23	4.92	4.96	4.96	4.91	4.91	4.92	4.91
24 (H,CH$_3$)	-.0.83	-0.84	-0.84	1.59-0.83	-.0.82	1.62-0.82	-.0.83
25	3.42	3.43	3.43	3.41	3.40		3.40
26	1.21	1.22	1.22	1.21		1.21	
27						1.02	
2'(H,CH$_3$)	2.43,1.17			2.58,1.17	2.57,-	2.57-1.17	-,1.17
3'		6.61	6.61	1.25,-			
4'	0.32			1.62	1.62	1.62	0.93
5'				0.91,0.88	0.91,0.88	0.91,0.88	

Example 2

The individual compounds were screened for their activity against the nematode *Caenorhabditis elegans*. The test system involves the suspension of the nematodes in a buffered, antibiotically attenuated *E. coli* containing nutrient medium containing a known concentration of the nematicidal compound. One week after suspension, the test units are examined under the microscope to assess the efficacy of the compound. The method is sensitive to a concentration of 0.01 ppm of ivermectin. The method is an adaption of that described by Simpkin (K G Simpkin and G C Coles, "The use of *Caenorhabdites elegans* for anthelmintic screening", J. Chem. Biotechr cl., 1981, *31*, 66-69). Nematode kills of 90% were caused by new compounds 4 and 5 at concentrations of 0.02 and 0.06 ppm respectively. In similar tests, the previously described milbemycins alphas 1 and 3 gave similar 90% mortalities at concentrations of 0.05 and 0.01 ppm respectively. Thus it may be concluded the new milbemycins 4 and 5 (and, by analogy, the other novel compounds) are of comparable biological activity to those previously described.

CLAIMS

1. A compound of formula (I) :



(I)

wherein R^1 is hydroxy, R^2 is hydrogen or methyl, R^3 is methyl, and R^4 is 2,4-dimethylpentenoyl or 2 4-dimethylpent-2-enoyl.

2. A compound according to claim 1 in substantially pure form.

3. A compound according to claim 1 wherein R^1 is hydroxy, R^2 is hydrogen, R^3 is methyl and R^4 is 2,4-dimethylpent-2-enoyl.

4. A compound according to claim 1 wherein R^1 is hydroxy, R^2 is hydrogen, R^3 is methyl and R^4 is 2,4-dimethylpentenoyl.

5. A process for preparing a compound of formula (I) as defined in claim 1 which process comprises cultivating a strain of streptomyces and isolating the compound of formula (I) from the fermentation mixture.

6. A process according to claim 4 wherein the Streptomyces strain is NCIB 11876 or a mutant thereof.

7. A process according to claim 6 wherein the Streptomyces strain is NCIB 11876.

8. A pesticidal composition comprising a compound according to claim 1 in combination with a carrier or diluent.

9. A composition according to claim 8 wherein the carrier or diluent is an agriculturally acceptable carrier.

10. A method of controlling or eradicating insects comprising administering to the insect or to an environment thereof a compound of formula (I) as defined in claim 1.

11. A compound substantially as hereinbefore described with refarence to the examples.

12. A process for preparing a compound of formula (I), substantially as hereinbefore described with reference to the examples.

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